

**Using Viral Induced Gene Silencing to Investigate a Methylation-Based Antiviral
Defense in Tomato**

A Senior Honors Thesis

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**by
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Background

RNA silencing is a natural regulation and defense system in many eukaryotic organisms, including plants, fungi, and animals. It was first discovered in plants when attempts to over-express a transgene copy of an endogenous gene led to the silencing of both genes. In this case, the transgenic over-expression targeted the transcripts of both genes for degradation (Baulcombe, 2002).

RNA silencing is triggered by double-stranded RNA (dsRNA) that can originate from many sources. These include secondary structure of an RNA transcript and the action of RNA dependent RNA polymerases (RDRPs) on aberrant (e.g. uncapped) transcripts generated by over-expression. The dsRNA is cut into 20-27 nucleotide (nt) small interfering RNAs (siRNAs) or micro RNAs (miRNAs) by a dicer-like protein (DCL), which enter one of several known RNA silencing pathways (Hamilton and Baulcombe, 1999). Three major pathways have been described in the model system *Arabidopsis thaliana*, with many related pathways specialized for particular functions. These include the miRNA pathway, the siRNA pathway (also known as RNA interference (RNAi) or post-transcriptional gene silencing (PTGS)), and the heterochromatin pathway, which can condition transcriptional gene silencing (TGS). In addition to the use of a DCL protein to start the pathway, the pathways commonly use an Argonaute protein to facilitate sequence recognition and slicing, or in some cases methyltransferase recruitment. Despite their similarities, however, the miRNA pathway, the siRNA pathway, and the heterochromatin pathway have distinct features.

The miRNA pathway is used as an endogenous regulator of gene silencing, especially of genes that control development or stress response pathways. These small RNAs are processed from partially folded stem-loop precursor RNAs that come from genes distinct from the genes they target. This is sometimes referred to as heterosilencing (Bartel, 2004). The miRNA precursor is targeted by DCL1, an RNase III homologue, to cut it into a mature, duplex miRNA of 21-22 nt. One strand of the miRNA associates with the Argonaute 1 protein (*AGO1*) within the RNA-induced silencing complex (RISC), which in plants is an miRNA or siRNA-directed endonuclease (Schwarz et al., 2004). This complex then uses the sequence specificity of the miRNA to degrade homologous target mRNA (Vaucheret, 2006).

The siRNA or RNAi pathway, also known as the post transcriptional gene silencing (PTGS) pathway, is a little broader. It encompasses both endogenous trans- or cis- acting siRNA as well as foreign dsRNA. The endogenous cis-acting siRNA is mostly derived from RNA transcripts from heterochromatin, transposons, and repeat elements. The trans-acting siRNAs (ta-siRNA) are processed from the TAS genes. The RNA transcribed from the TAS genes is long non-coding RNA that seems to function only as a precursor to the ta-siRNAs. Importantly, foreign dsRNA can also be part of this pathway. In most instances, foreign dsRNA is generated from the genomes of invading RNA viruses, or transcripts of DNA viruses. The dsRNA is cut into 21-22 nt siRNA by DCL2 or DCL4 and at that point acts similarly to the trans- and cis- acting siRNAs. Any of these small RNA variations can then complex with *AGO1* in RISC, leading to host mRNA cleavage (Vaucheret, 2006).

The heterochromatin pathway is one that acts on DNA and can cause transcriptional gene silencing (TGS). This pathway is activated by siRNA, but acts on homologous DNA. As an endogenous system, it acts to maintain methylation levels on promoters and coding sequences of transposons and repeat sequences, creating inaccessible heterochromatin (Qi et al., 2006). Methylation of promoters lowers transcription levels of the gene and also serves to recruit components of heterochromatin. In this pathway, the siRNA is processed through DCL3. This dicer is similar to the dicers of the previously mentioned silencing systems, but cuts the dsRNA into siRNAs of 24 nt. One strand then associates with an Argonaute 4 protein (*AGO4*) as part of a methylation complex that recruits cytosine and histone methyltransferases. In the case of foreign DNA, the DNA is methylated and remodeled so that it is not transcribed.

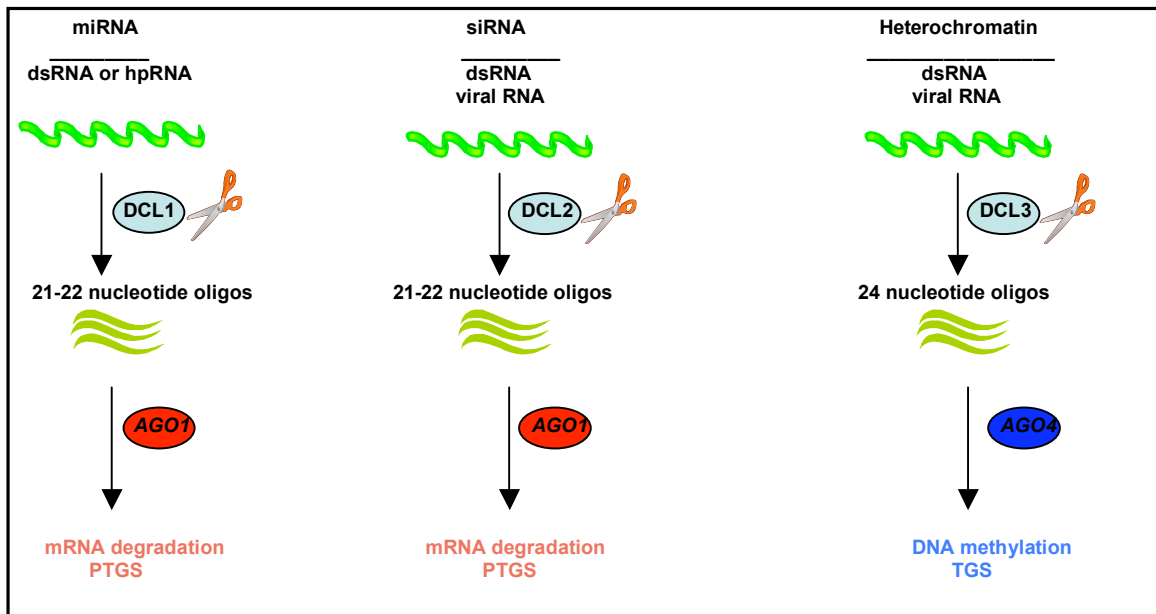


Figure 1: RNA silencing pathways: There are three known pathways of RNA silencing. All are triggered by dsRNA, which is processed by a DCL into small siRNAs or miRNAs. The miRNA pathway is an endogenous regulation pathway. The RNAi pathway can be an endogenous system or a defense system against foreign nucleotides. Both pathways use the RISC complex with *AGO1* as an endonuclease, which degrades RNA homologous to the oligos formed by the DCL. The heterochromatin pathway is different in that instead of acting as an endonuclease, the RISC complex with *AGO4* recruits DNA methyltransferases.

As alluded to already, these RNA silencing pathways are important for more than regulation; they are a defense against foreign DNA, including that of geminiviruses (Bisaro, 2006; Raja et al., 2008). The Geminiviridae is a large plant virus family that has circular single stranded DNA (ssDNA) genomes. These viruses rely on the host replication machinery to replicate, as they do not code for or carry their own DNA polymerases (Castillo et al., 2004). During replication, geminiviruses go through a double-stranded DNA (dsDNA) phase, associate with histones, and form mini-chromosomes (Pilartz and Jeske, 1992). The dsDNA intermediate acts as template for both viral replication and transcription. The transcripts produced are subject to silencing through PTGS and may also stimulate methylation of the minichromosomes to cause TGS (Bisaro, 2006).

One of the most obvious indicators that RNA silencing is an antiviral defense is the presence of silencing suppressors encoded in the genomes of the majority of viruses (Li and Ding, 2006). This suggests that host defense and viral gene regulation have exerted an influence on each other evolutionarily (Voinnet, 2005). In addition, the suppressors have been shown to be virulence factors for the viruses, enhancing replication and spread. The first silencing suppressor discovered was HC-Pro, which proved to enhance the replication of viruses unrelated to the potyvirus from which it

originated. It was effective in increasing the stability of the dsRNA as a silencing trigger, suggesting inhibition of DCL function (Voinnet, 2005). Following the discovery of HC-Pro as a suppressor, many other suppressors were found, acting through a variety of mechanisms. In geminiviruses specifically, the AL2 (also known as AC2) protein has been found to be a suppressor of silencing along with the L2 (C2) protein, which shares some homology with AL2 (Voinnet et al., 1999, Wang et al., 2005). In addition, the L4 (C4) proteins of at least some geminiviruses can also suppress silencing (Vanitharani et al., 2005). These silencing suppressors in general work to enhance susceptibility of the host while increasing the replication and virulence of the virus.

The fact that viruses induce an RNA silencing response has been taken advantage of in a method called virus induced gene silencing (VIGS) (Ruiz et al., 1998). By using a virus vector that stimulates the silencing response, a gene of interest can be inserted and included in the RNA that is silenced. As mentioned before, a double-stranded RNA (dsRNA) signal is generated during virus replication, activating the silencing complex which leads to the processing of siRNAs from longer dsRNA. These siRNAs then guide RISC to degrade any RNA with homologous sequence (Chicas and Macino, 2001). This causes not only viral RNA to be silenced, but also mRNAs from the gene of interest to be silenced as well.

Many different VIGS vectors have been used successfully, but an ideal vector has not been found. The first widely effective vector used in VIGS was *Tobacco rattle virus* (TRV), which is a bipartite, positive strand RNA-containing tobnavirus. The TRV genome was modified to have a multiple cloning site within it, replacing non-essential genes. Other RNA virus vectors that had been used for VIGS include *Tobacco mosaic*

virus (TMV) and *Potato virus X* (PVX) (Ratcliff et al., 2001). However, each of these vectors has limitations. First, the VIGS phenotype can be difficult to distinguish over a background of chlorosis and necrosis that virus symptoms produced. Second, most viruses cannot invade every cell or the growing points (meristems) of plants. Thus some cells were unaffected, and it was difficult to study genes affecting development (Ratcliff et al 2001). The TRV vector was able to overcome some of these limitations by having very mild symptoms, infecting many cells, and having the ability to infect growing points (Ratcliff 2001). The disadvantage of this vector is that it is an RNA virus, which causes the virus genome itself to be targeted by the silencing systems. This causes eventual clearing of the virus, leaving long term data unattainable. Also, its bipartite genome creates additional work during cloning steps.

Since the construction of TRV, several geminiviruses have been constructed as VIGS vectors, including *Tomato golden mosaic virus* (TGMV) and *Cabbage leaf curl virus* (CaLCuV), which are relatively distantly related to each other (Turnage et al 2002). Both of these viruses have been used successfully in silencing. They have some advantages over RNA virus vectors, since their DNA genomes are not targeted for degradation, and they produce relatively strong systemic silencing signals that can penetrate the meristem. But they also have limitations. The first limitation is one of size. Geminiviruses have a limit on the amount of DNA that can be packaged, and encapsidation of the viral genome is necessary for systemic infection. In practice this limits insert size to 1 kb or less. When this limit is approached, the virus can no longer effectively package and may lose the insert more frequently (Burch-Smith et al 2004). In addition to the size limitation, the TGMV and CaLCuV-based vectors cause a

background of viral symptoms similar to those attributed to TMV and PVX. Also, both have a bipartite genome, and for ease of use, monopartite would be better. Another unfortunate limitation is the relatively narrow natural host ranges of these two viruses. TGMV infects members of the *Solanaceae* (tobacco, tomato, etc.) whereas CaLCuV infects members of the *Brassicaceae* (cabbage, Arabidopsis, etc.).

An ideal VIGS vector would be one that did not need to package and spread to transmit the silencing signal. This would eliminate insert size restrictions as well as symptom background. Also, to make it widely applicable, the virus on which it is based should have a broad host range. Last, in an effort to make it easy to use a monopartite virus is superior to a bi- or multi-partite genome virus. This was the goal in the construction of the *Beet curly top virus* (BCTV)-based vector that is used in our silencing experiments.

BCTV is a monopartite geminivirus belonging to the genus *Curtovirus*. It is reported to have a very broad host range, consisting of over 40 families of plants, making it widely applicable in the realm of agriculturally useful crop species. In the construction of the vector, the virus coat protein and other genes required for the virus to spread were disabled, preventing movement of the virus from the inoculation site. The R1 and R2 genes were both truncated in the vector and have stop codons introduced early in the remaining sequence. They are responsible for synthesis of the capsid protein and accumulation of viral ssDNA, respectively. However, the gene required for replication, L1, is still intact. This construction allows for the replication, but not spread of the virus, separating silencing phenotypes from viral symptoms. Evidently, BCTV replication generates a strong systemic silencing signal that causes endogenous genes to be silenced

without spread of the BCTV vector itself. The fully constructed virus is named pWSRi (Golenberg et al., unpublished). The pWSRi vector can replicate in *E. coli* due to its pUC plasmid backbone. However, in plant cells, the BCTV vector escapes by recombination via repeated sequences (recombination sites) at its ends and replicates as a viral chromosome.

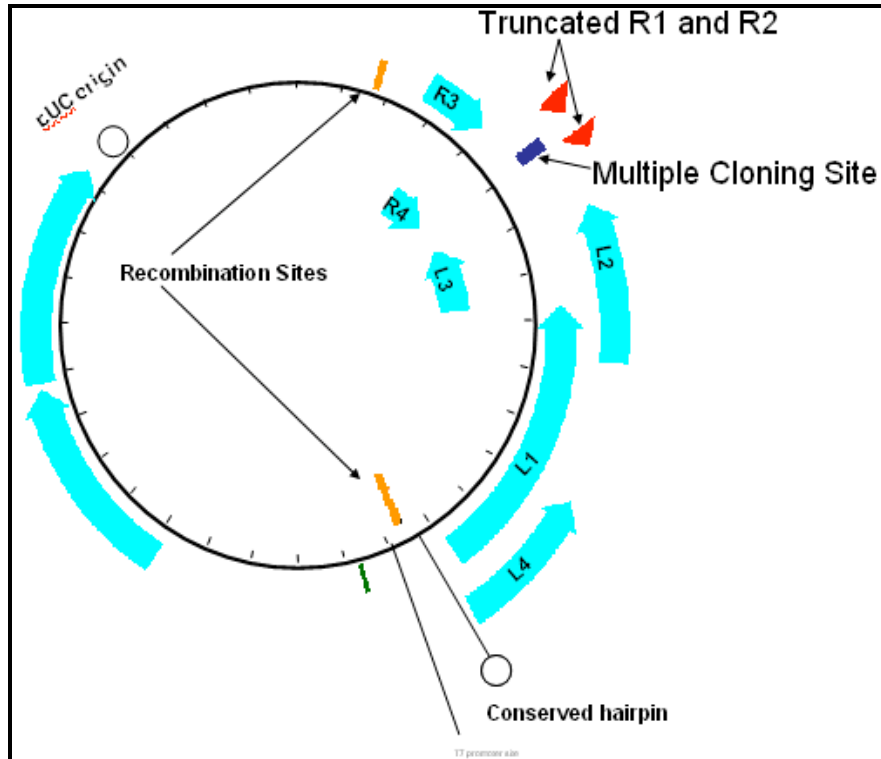


Figure 2: The BCTV VIGS Vector: pWSRi. The vector is composed of the BCTV genome inserted into a pUC based vector. The genome has been altered to have truncated R1 and R2 genes to prevent spread of the virus when it recombines out of the pUC vector via the recombination sites marked. The R1 and R2 genes also have premature stop codons. The L1 gene is the only gene necessary for replication, and it is functional. The conserved hairpin which is part of the origin of replication is within the margins of the recombined virus genome.

This vector was first tested in cultivated spinach to silence a *rbcS* gene (ribulose biphosphate carboxylase, small subunit) and a transketolase gene. Silencing of these genes produces a bleaching phenotype, as they are required for the Calvin cycle. The

method of inoculation was by biolistic bombardment, an effective, but somewhat complicated method. It involves precipitation of DNA onto tungsten beads. These beads are shot at the plants with a particle gun, delivering the DNA through the wounds inflicted by the beads. The bleaching occurred systemically in the plants treated biolistically with the pWSRi vectors that contained inserts. Empty vector and other control plants were unaffected. In addition to the *rbcS* and *transketotase* genes, genes expressed in the floral meristem were used in silencing experiments. Silencing of these genes caused abnormal flower formation. In spinach, only male flowers or only female flowers exist on an individual plant. Genes involved in the formation of male plants were silenced, producing female phenotypes or a combination of male and female flowers. Female plants had no change when bombarded with the silencing treatments, as those genes are not applicable in female flower formation (Golenberg et al., unpublished).

This work in spinach has led to my work in *Solanum lycopersicum*, or tomato. I have endeavored to use the novel BCTV vector in a new host, tomato, and with a new inoculation method, agrobacterium infiltration. Using this novel vector in silencing experiments, the goal is to investigate the genes involved in the tomato heterochromatin pathway and which also lead to methylation defense against geminiviruses. First however, the BCTV vector was tested with genes with which silencing could be easily visually detected. By using a magnesium chelatase (*Su*) gene and a phytoene desaturase (*PDS*) gene, we have been able to test the effectiveness of the BCTV vector. Silencing was detected by observation of bleaching. RNA analysis in the form of RT-PCR was performed to confirm the reduction of target RNA in the samples in which silencing was visually detected.

The next step in this work is to begin to investigate the role of methylation in viral defense. Previously, our lab has investigated the role of different genes in the heterochromatin or methylation pathway of *Arabidopsis thaliana*. Our lab has discovered that plant hosts methylate cytosine residues in geminivirus genomes in order to inhibit viral gene expression and replication. An important proof of this defense is that *Arabidopsis thaliana* plants that have mutations in genes important for methylation are hypersusceptible to geminiviruses.

My hypothesis is that tomato uses methylation as part of a viral defense system as well. To test this, *in silico* work has been done to find sequences of genes homologous to those studied in *Arabidopsis thaliana*. These will then be used in the BCTV VIGS vector to create knock-out phenotypes. Super-infection with other distantly related geminiviruses will allow analysis of any hyper-susceptibility, providing evidence for involvement in the methylation pathway. At the same time, this work should identify genes involved in the tomato heterochromatin pathway. This project will serve to translate research that has been done on a model organism to organisms that are important in agriculture and useful to understand.

Materials and Methods

Cloning Su and PDS into the BCTV Vector

The *Su* gene was obtained from vector pTV:09 (obtained from D.C. Baulcombe, The Sainsbury Laboratory, Norwich, UK) by PCR. The *Su* gene was then inserted into a T-vector and sequenced. The Arabidopsis *PDS* gene, AT4G14210, was obtained by PCR of ABRC clone UO9151. It was also cloned into a T-vector and sequenced.

The *Su* and *PDS* genes were then inserted into the BCTV vector pWSRi, a pBI 121 based binary plasmid containing a 1.5-mer of the viral genome (Golenberg et al., unpublished). The BCTV vector has deletions in the R1 and R2 genes, which are responsible for the coat protein and accumulation of single stranded DNA. These genes are required for movement, and deletion of them prevents the virus vector from packaging and spreading in the host plant. The L1 gene, required for replication, is functional and intact.

The pWSR1:Su and pWSRi:PDS vectors, as well as control vectors, were transformed by electroporation into *E. coli* strain DH10B for screening, and later transformed into *Agrobacterium* strain C58c1 for agroinfiltration.

Creating an L4 mutant derivative vector

This vector was constructed by and obtained from Dr. Kenneth Buckley, the postdoctoral associate with whom I have been working. A premature stop codon was inserted into the L4 gene, a known silencing suppressor, to truncate any expressed protein (Vanitharani et al., 2005). While the L4 and L1 genes overlap, the mutation in the L4

gene does not affect the L1 gene. Vectors with an L2 mutation were also constructed, as well as vectors with an L2/L4 double mutation. These vectors have not yet been tested.

Agroinfiltration of tomato

The BCTV vector and derivatives were transformed into *Agrobacterium* strain C58c1. To prepare each infiltration cultures were grown overnight at 30°C in the presence of antibiotics and 20µM acetosyringone. The cultures were resuspended to OD₆₀₀ of 1 in a buffer containing MgCl₂, MES, and acetosyringone. The resuspended cells were allowed to sit in the buffer for 3 hours before infiltration.

The acetosyringone allows the *Agrobacterium* to transfer the DNA more effectively to the plant cells. In nature, it is a compound that plants secrete, and to which *Agrobacterium tumefaciens* is attracted. It signals the *Agrobacterium* that a plant can be colonized. It also prepares the bacterium for injection of DNA into the plant cells by inducing the activation of virulence genes (Pappas and Winans 2003).

To agroinfiltrate the plants, a needle-less syringe was used to press *Agrobacterium* cultures into the underside of the cotyledons or leaves until a spreading stain was observed. If at the cotyledon stage, both cotyledons were infiltrated. At the leaf stage, 3 leaves were infiltrated.

RNA analysis of silenced tissue

Tissue with silencing symptoms was harvested and flash frozen in liquid nitrogen. 200 mg of tissue was collected for each sample. The tissue was then ground and put through an RNA extraction and purification using Trizol TM.

The levels of Su mRNA and control ribosomal RNA (rRNA) were analyzed using an Invitrogen one step RT-PCR kit, following the kit protocol and using 1 µg of purified RNA. The sequences of the oligonucleotide primers for the internal control (rRNA) were:

5':cctgggttgatcctgccagtag
3':accaactaagaacggccatgc

The Su primer sequences used were:

5':gtagggcaagatgagatgaag
3':tgctcttgctgctctgtagt

Argonaute alignments

Nucleotide sequences of the Argonaute 4 protein were found for both *Arabidopsis thaliana* and *Solanum lycopersicum* on the TAIR database and the Sol Genomics database, respectively (TAIR: <http://www.arabidopsis.org/> and Sol Genomics: <http://www.sgn.cornell.edu/>) These sequences were aligned using Clustal W and conserved sequences were noted and differentiated from unique sequence.

Results and Discussion

Due to the success of the BCTV vector in spinach, we decided to use it in experiments in tomato. The tomato genome is not completely sequenced (currently about 30% complete), nor are there knock-out mutants that are widely available. This makes VIGS an important tool for deciphering the roles of different gene products in this organism, and others where characterized mutants are unavailable. Our hope is that the BCTV vector will be effective in creating knock-out phenotypes in tomato and other organisms in which knock-outs are not readily available. In these experiments we endeavored to show that the vector could be effective in a new host (tomato) as well as with a new inoculation method (agroinfiltration) in addition to the biolistically inoculated spinach that has already been tested.

Silencing of *Su* gene with the BCTV vector

Using the cloning methods discussed earlier, we inserted the *Su* gene, a magnesium cheletase gene, into the BCTV VIGS vector. It was transformed into *Agrobacterium* for inoculation. In the first experiment, only one plant was used as a preliminary trial. It was inoculated at the cotyledon stage to allow for effective spread of the silencing signal throughout the growing tissue. Approximately 3 weeks later, evidence of silencing began to appear in the form of bleaching. Multiple leaves were affected by this phenotype. Within a week, many leaves showed signs of bleaching. Some leaves were completely bleached, others showed incomplete bleaching phenotypes.

In subsequent experiments, a larger sample size was used along with a *pWSRi:CAT* control and a mock *Agrobacterium* control. The *pWSRi:CAT* control is the

pWSRi vector with a CAT (chloramphenicol acetyl transferase) gene inserted into the cloning site. The CAT gene confers resistance to chloramphenicol, which is irrelevant in the tomato system. No silencing phenotypes were expected with this control. The mock *Agrobacterium* control is strain C58 without the *pWSRi* vector. Again, no silencing phenotype was expected. In four independent experiments with a total of fifteen *pWSRi:Su* inoculated plants, 80% (12 of 15 plants) exhibited bleaching indicative of silencing (Table 1). None of the sixteen plants treated with the *pWSRi:CAT* control exhibited any bleaching, and neither did any of the mock controls. The silencing phenotypes for all of the affected plants begin to appear within 3-4 weeks, becoming more apparent and severe with time. Past this point, it is hard to give accurate observations, as the plants became sickly, masking the silencing symptoms. This sickness was not due to virus symptoms, but likely was a product of the bleaching phenotype affecting the metabolism of the plants.

In most cases, while the bleaching was not uniform or readily predictable, multiple leaves were affected and silencing phenotypes were easily distinguishable from a wild type or control plants. The characteristic silencing pattern can be seen in Figure 3. Silencing typically starts at the petiole and midvein and spreads distally through the leaf. This pattern, which indicates that silencing signals spread from the phloem into the mesophyll, is one way that silencing can be distinguished from other types of unrelated chlorosis.



Figure 3. Phenotypes of *pWSRi:Su* silencing compared to *pWSRi:CAT* control. A) A *pWSRi:CAT* inoculated control. It shows no visible silencing phenotype, as expected. B) A *pWSRi:Su* inoculated plant. It shows severe evidence of silencing. The bleaching is not uniform, but is easily distinguished from the control plant. It can be seen in this picture that the bleaching begins in the center of the leaf at the petiole and midvein and spreads distally through the leaf.

Not all of the trials were successful. As Table 1 shows, in one experiment, none of the 8 plants treated showed signs of silencing. In this trial, we were attempting to optimize conditions for the growth of the plants themselves. This included an increase in the temperature from 24°C to 27°C. This temperature rise seems to inactivate silencing, suggesting that a component of the tomato silencing machinery is temperature sensitive. The data from this trial (experiment 4) was included in the table, but was not in calculating the average silencing percentage. Observation of a temperature effect suggests that silencing phenotypes might be enhanced by optimizing plant growth conditions. In future experiments, the effects of temperature and light conditions on silencing will be investigated..

Infiltration	# plants infiltrated with <i>pWSRi:Su</i>	# with silencing	% Positive
1	1	1	100
2	4	4	100
3	6	5	83
4*	8	0	0
5	4	2	50

Table 1. Percent of inoculated plants that presented silencing phenotypes. The average success rate with this vector was 80%, or 12 of 15 plants. In experiment number 4, none of the plants showed evidence of silencing. This might be attributed to temperature sensitivity. The plants in infiltration 4 were grown at 27°C while all others were kept at 24°C. The data from experiment 4 was not included in the average silencing percentage.

Silencing of *PDS* gene with the BCTV vector

Silencing of *phytoene desaturase* (*PDS*) also results in bleaching phenotypes. We inserted this gene into the BCTV VIGS vector again using the cloning procedures that are discussed above. In addition, a new variation on the vector was used. In this derivative, the L4 gene, which is a known silencing suppressor, was mutated to render it non-functional. Both the wild type vector (WT), *pWSRi:PDS*, and the L4 mutant vector, *pWSRi:PDS L4*, were used to inoculate tomato by agroinfiltration. With both vectors, bleaching began to appear by 3-4 weeks after infiltration. In this case, both the *pWSRi:PDS* vector and the *pWSRi:PDS L4* vector had a silencing rate of 50%. In each of two experiments, 2 out of 4 plants showed silencing (Table 2). This suggests that mutating the L4 silencing suppressor has no effect on the overall silencing system.

There was no difference between the two vector derivatives in the severity or the uniformity of the silencing phenotypes. This is a counter-intuitive result. The expected

result is one in which silencing is more severe or at a higher occurrence in the L4 vector. This was not the case. This could be because of the dual roles that silencing suppressors play. While it is true that they reduce the spread of the silencing signal, it is also true that they increase the replication efficiency and copy number of the virus. Thus, while the spread of the silencing signal might be increased by mutation of the suppressor, the copy number and replication efficiency of the vector might also be lowered. This creates a situation in which the overall system is unchanged. Unfortunately, the sample size in this experiment is small, so definitive conclusions cannot be drawn. In future work, more trials will be done with this derivative vector and other derivative vectors that are currently being assembled. One will have a mutation in the L2 gene, another silencing suppressor, while another will have mutations in both the L2 and the L4 genes.

Phenotypically, the bleaching with *PDS* was very similar to that of the *Su* gene, except that bleaching appears more yellow than white. The timing and extent of silencing phenotypes observed was about the same with both genes. Again, the bleaching characteristically began at the petiole and midvein, spreading distally throughout the leaves. The silencing symptoms were not uniform through the whole plant, but they affected more than a single leaf at any time. In Figure 4, it can be seen that all of the leaves on one section of the plants were affected.

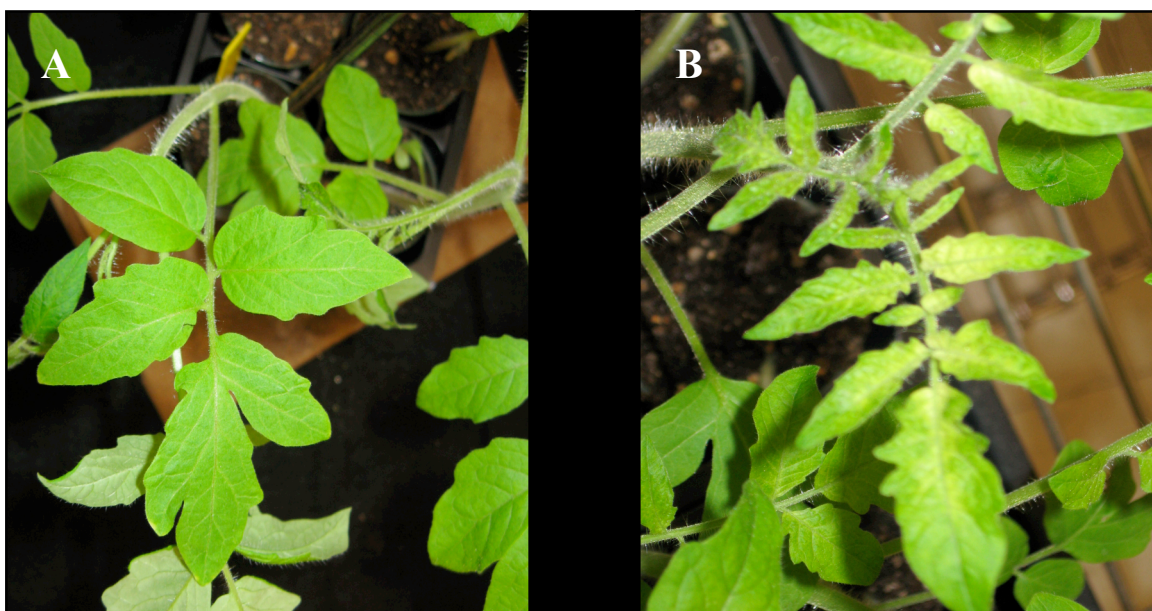


Figure 4. Phenotypes of *pWSRi:PDS* silencing compared to *pWSRi:CAT* control. A) *pWSRi:CAT* inoculated control. It shows no visible silencing phenotype, as is expected. B) A *pWSRi:PDS* inoculated plant. It shows evidence of silencing. The bleaching is not uniform, but is easily distinguished from the control plant. It can be seen in this picture that the bleaching begins in the center and spreads distally through the leaf. The bleaching occurs approximately 3-4 weeks after inoculation.

Infiltration	# plants infiltrated	# with silencing	% Positive
pWSRi : PDS	4	2	50
pWSRi : PDS L4	4	2	50

Table 2. Percent of inoculated plants that presented silencing symptoms. The average success rate with this gene was about 50%, or 4 of 8 plants. The two vector derivatives used were pWSRi:PDS and pWSRi:PDS L4. In the second variation, the L4 gene is mutated so that it will be non-functional. L4 is known as a silencing suppressor.

RNA analysis of silenced tissue

While visually, the experiments performed with the pWSRi:Su and pWSRi:PDS vectors appeared to be successful, analysis of the RNA levels is being performed to confirm the results at the molecular level. It is expected that endogenous Su and PDS mRNA levels will be reduced in silenced tissues.

Tissue from leaves that displayed silencing phenotypes was collected and flash frozen, and RNA extracts were prepared as described in Materials and Methods. The RNA was then analyzed by semi-quantitative RT-PCR (reverse transcriptase-PCR) using primers constructed from the *Su* gene and ribosomal RNA (rRNA) primers as an internal control. Unfortunately, in an initial experiment the two sets of primers interfered with each other, resulting in amplification of a DNA fragment corresponding to *Su* mRNA but not the internal control rRNA. This made the RNA levels difficult to compare and necessitated a move to doing each reaction individually. Subsequently an RT-PCR experiment was carried out using only the *Su* primers. Consistent with visual results, the sample from the unsilenced tissue of the *CAT* control had the highest level of transcript, while the RNA extracts from the silenced tissue had less *Su* mRNA. In a third trial, the ribosomal RNA control and the *Su* amplification reactions were done simultaneously in separate tubes. All reactions were done under the same conditions at the same time. In this reaction, there was no discernible difference in the *Su* mRNA RNA levels between silenced and unsilenced tissues. In future work, new ribosomal primers will be used to try to conduct the experiments in the same tube without interference of the primer sets. Also, fewer cycles will be done to keep the reactions from reaching saturation. By doing this, it will be easier to detect differences in mRNA levels.

***In silico* alignments of *Argonaute* sequences**

After the vector was found to be useful and effective in silencing endogenous genes in tomato, the focus turned to finding genes of interest in the methylation pathway. In *Arabidopsis thaliana*, a specific member of the *Argonaute* gene family (*AGO4*) has been shown to play a key role in the methylation-directed viral defense pathway. To expand on these findings and apply them to tomato, the goal is to create *AGO* knockout phenotypes. To do this, I have aligned tomato *AGO* gene and protein sequences with those of *Arabidopsis*. Unfortunately, the whole tomato genome has not been sequenced yet, making this task difficult. However, an *AGO4* sequence is available and enough genome has been sequenced that the project can be initiated. By searching for tomato unigene sequences similar to *Arabidopsis* Argonautes on the Sol Genomics database, multiple homologous sequences were found. These sequences were aligned to search for conserved areas within *AGO4* and the Argonaute family as a whole. Useful sequences for gene knock-down are ones that are conserved within the *AGO4* genes of multiple organisms, but are distinct from other genes in the Argonaute gene family. By making them specific to *AGO4*, we hope to avoid knocking-down multiple *AGO* family members.

We also prefer to use sequence that is conserved among *AGO4* genes in other species to provide a platform for wider application than just tomato. These sequences will be used in the BCTV vector in future work to investigate the role of *AGO4* protein in the methylation pathway. Knock-downs of these genes will be created with the BCTV VIGS vector, and later super-infected with other relevant geminiviruses. If our hypothesis is correct, the *AGO4*-silenced plants will have greater susceptibility to these

viruses. The knock out will disrupt the methylation (heterochromatin) pathway of RNA silencing, lowering the defense mechanisms that the plant usually uses to fight off an infection.

Argonaute proteins have two large, highly conserved domains, the PAZ domain and the PIWI domain. These regions are obvious and easily detected by alignment of the protein sequences, as Figure 5 shows. The sequences at the nucleotide level, however, are much less conserved. This is important because the nucleotide sequence is what will be useful in silencing experiments.

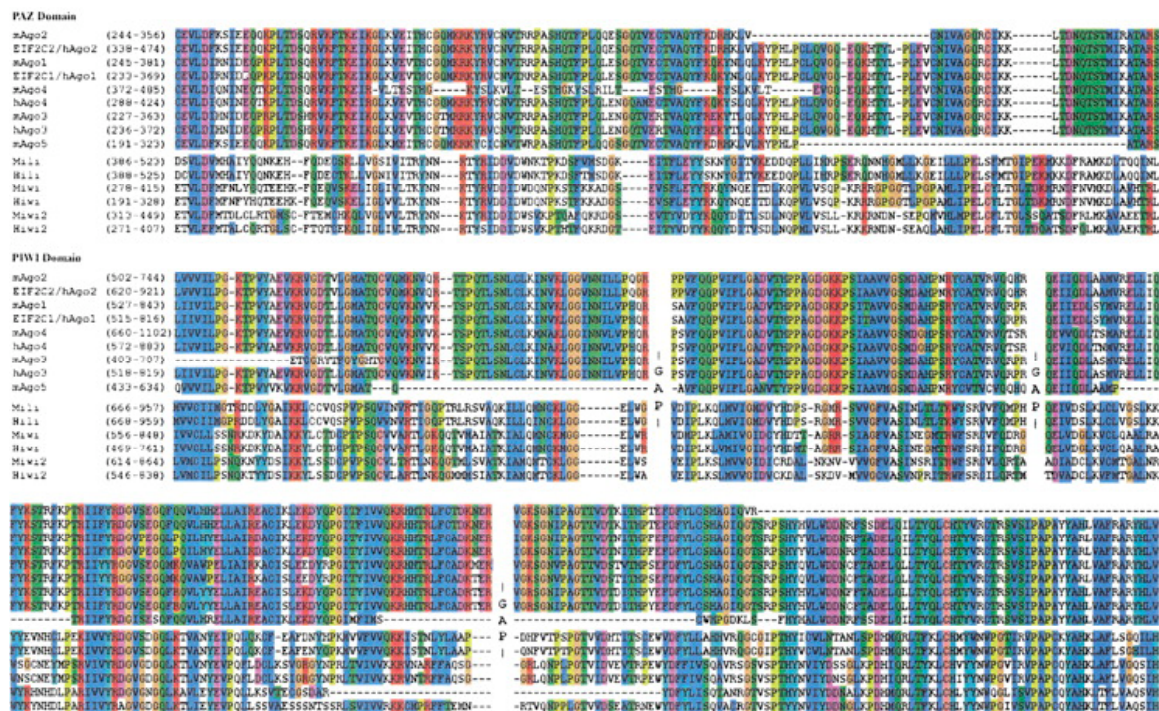


Figure 5: The Conserved PAZ and PIWI Domains in the Argonaute gene Family. Carmell, M.A. et al 2002. AGO proteins from human, mouse, and Arabidopsis were aligned to show the conserved domains.

When Arabidopsis Argonaute family members were aligned by nucleotide sequence, only three of the genes had relevant homology, *AGO4*, *AGO6* and *AGO7*. *AGO7* has only marginal homology (8%) with *AGO4*. In an experiment to silence *AGO4*

then, *AGO6* is really the only other family member that poses a concern. The two genes exhibit over 50% homology, with some regions more conserved than others. It is assumed that similar genes also exist in tomato, although at this time complete sequences are available only for *AGO1* and *AGO4*. An alignment of Arabidopsis and tomato *AGO4* genes shows that they are highly similar (60%) (Figure 6). Figure 7 shows a region that would be problematic in a silencing experiment because of the high homology between tomato *AGO4* and Arabidopsis *AGO6*. Figure 8 shows a region that may be useful in silencing because it has low homology with Arabidopsis *AGO6*, but is conserved among other *AGO4* genes in other species, which fulfills the criteria for a good silencing sequence. Multiple silencing sequences of 30 to 40 nucleotides can be found in this region to confirm that the silencing phenotypes seen are a result of silencing only *AGO4*.

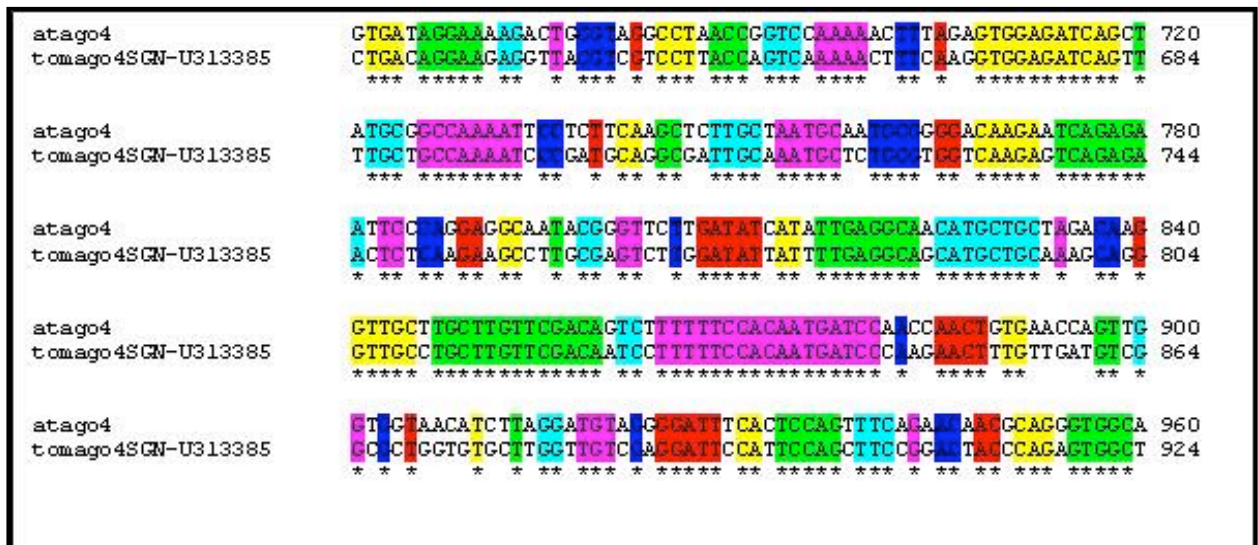


Figure 6. Alignment of Arabidopsis *AGO4* and tomato *AGO4*. The two *AGO* genes are reasonably conserved at the nucleotide level as well, showing that silencing with this sequence could be useful. It may even be possible to use the same sequence to silence the gene in both organisms.


```

atago6      ACCACACCAAGTTGTTTACGTTAAGGCTCCTGAAATGTTTCCTGACAAAGTGTCTGCG 2447
tomago4SGN-U313385 ATCATACCAAGTTTTCCTGCTAATGATCTTAACTATGTTTCCTCTAGTCAATCATAG 2505
* ** * * * * * * * * * * * * * * * * * * * * * * * * * *

atago6      ACACCAAGATGTAACCCCGCAAACTACCTTTTAAATGTGTGCTCATGCAAGAAAA 2507
tomago4SGN-U313385 ACAACAAGTCTGTCAACCAAGGAATATCTTTTAACTGTGTGCTCATGCTGGAATGA 2565
*** ** * * * * * * * * * * * * * * * * * * * * * * * * *

atago6      TAAACATTCAAAACCGGCTCATACCATGTATTACTTCATGAGATTCTTTCTCCCG 2567
tomago4SGN-U313385 TTCTTCAAACTCTCTTACTCAGATATACGTCTTGTATCATGAGCTGCTTTCTGCTG 2625
* ** * * * * * * * * * * * * * * * * * * * * * * * * *

atago6      ATGACTTGCAGATTCTCATCCATTCTCTCTCTATCTCACTACCTAGCACAACTGCAG 2627
tomago4SGN-U313385 ATGATCTTCAAGAGCTTGTTCATAATCTCTCTTATGTGTCTGAGAAGCAACCCTGCAG 2685
**** * ** * * * * * * * * * * * * * * * * * * * * * * *

atago6      CTTCGATCTGCTCCAGTACCATACCTCATTTCTACAGCTCAAGTTCCGCAATCA 2687
tomago4SGN-U313385 TATCTGTTCTCTCTTATCTCTTATCCCATCTCTCTCTTACTCAAAATGGACAGGCA 2745
** * ** * * * * * * * * * * * * * * * * * * * * * * *

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Figure 7: Highly conserved sequences between tomato *AGO4* and Arabidopsis *AGO6*. This stretch of sequence would not be useful in a silencing experiment, because it would most likely produce a knock down of both *AGO4* and *AGO6*, which is not desired.

```

atago6      ACTGTGA---CGGACAAGCAAAAGAGTAAATAA-ATTCCTCTCTTTATTACCGGA 87
tomago4SGN-U313385 TCTCCTCTCTTSCATTAGGATTTTCTTCTGATTTTGGTCTTCATCTCCTTTTGA 120
** * * * * * * * * * * * * * * * * * * * * * * * *

atago6      ACCTACCAATCCCTCTTAATTCTTCAAAAGCA-----TATTTTCCTGCAAGGAT 141
tomago4SGN-U313385 CAGAACATAGGATCTGATATGGCTGAAGAAGAAACAAATGGAGCAGAGCAAGGCTC 180
* *** * * * * * * * * * * * * * * * * * * * * * * *

atago6      CAGACATCTTATTC-TGCCACTG---TCACCA---AGTATAGAACAGAACAG 193
tomago4SGN-U313385 TCCACCTCCCTCCCTGTTCACACAGATTTCACCTCCGCTAAACTGAAATTGAGCCTC 240
* ** * * * * * * * * * * * * * * * * * * * * * * *

atago6      CCCACCTTCGAGATTATCAGATCA---ATAGACCTGCGCTTGGAAACCAACAAC 251
tomago4SGN-U313385 TGAATAAAGATCTTCCCGTTCCATGGCAGCGCTGCTTTGCAAAATAGACAAA 300
** * ** * * * * * * * * * * * * * * * * * *

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Figure 8: Significantly different sequence between *AGO6* and tom *AGO4*. These would be useful in silencing experiments due to the significant differences in sequence. Most likely only one or the other would be knocked down depending on the sequence used, but not both.

Conclusion

The novel VIGS vector based on BCTV has been shown to be effective in silencing genes in tomato on the visual level. A high percentage of plants that were treated with either *pWSRi:Su* or *pWSRi:PDS* derivatives have shown convincing signs of silencing in the form of a bleaching phenotype, while those treated with control vectors did not. Although RNA data is still yet to come, the visual data has been encouraging. This shows that the vector has been successful in a new host and with a new inoculation method.

One interesting finding is that the mutation of the L4 gene did not effect silencing even though it is thought to be a silencing suppressor. More trials with a larger sample size will need to be done to come to concrete conclusions. Another interesting finding was the apparent temperature sensitivity of the tomato silencing system, which also will need to be further investigated.

To continue investigating viral defense in tomato, Argonaute sequences have been found that will be useful in silencing Argonaute 4. Plants in which *AGO4* expression has been knocked down will be super-infected with distantly related geminiviruses to detect any hyper-susceptibility, an indication of involvement of *AGO4* in the heterochromatin silencing pathway of tomato and its use in viral defense.

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